

STUDIES ON THE QUANTITATION OF INTRAOCULAR *Treponema pallidum**† AN IN VITRO MODEL

BY

FRANCIS W. CHANDLER, JR., AND LESLIE C. NORINS

Venereal Disease Research Laboratory, Venereal Disease Program,

National Communicable Disease Center,

Health Services and Mental Health Administration,

Public Health Service,

U.S. Department of Health, Education, and Welfare, Atlanta, Georgia 30333, U.S.A.

Smith and Israel (1967a, 1967b, 1968) and Goldman and associates (Goldman and Girard, 1968; Girard, Goldman, Baumstark, and Stickle, 1967) have recently reported that spiral organisms, some of which are motile, can be demonstrated in the aqueous humour (AH), cerebrospinal fluid (CSF), and tissues of adequately treated syphilitic patients. Most of these organisms are morphologically indistinguishable from *Treponema pallidum* and can be demonstrated by direct immunofluorescent techniques. These investigators strongly suggest that these spiral organisms, some of which have been transferable to animals (Smith and Israel, 1968), are *T. pallida*.

Recently, Chandler and Cannefax (1969) have established that immunofluorescent techniques can be more efficient than darkfield microscopy for the demonstration of *T. pallidum* in body fluids. *T. pallidum*-like organisms thus far reported in ocular fluids have been very few in number when detected by either darkfield microscopy or direct immunofluorescent staining. Clinical studies to date have been qualitative in nature, and a method to relate the number of treponemes observed on the final direct fluorescent antibody (FA) slide to the approximate number of organisms per unit volume of AH would be highly desirable in order to establish an initial base line. Against this base line the evolution of infection may be measured and the response to therapy assessed. It would further benefit the investigator to have data indicating the smallest number of treponemes that could reasonably be detected per unit volume of fluid when direct immunofluorescent techniques are used. This paper

reports an *in vitro* model for the experimental quantitation of intraocular *T. pallidum*.

Materials and Methods

Preparation of Test Suspension

Source A suspension of *T. pallidum* (Nichols strain) was prepared by extraction from acute rabbit testicular syphilomas as described for the *T. pallidum* Immobilization (TPI) test (U.S. Public Health Service, 1964), with 0.15 M NaCl being substituted for the usual TPI test medium. Centrifugation of the extract for 5 min. at 650 relative centrifugal force (RCF) freed the supernatant fluid of gross cellular debris. The supernatant fluid was decanted and recentrifuged for 20 min. at 1,375 RCF, and the sedimented treponemes were then resuspended in rabbit aqueous to serve as the test suspension for later dilution and quantitation.

Enumeration The number of organisms per unit volume of test suspension was determined by the dark-field enumeration method of Morgan and Vryonis (1938), which is based on knowing the volume of one microscopic field under well-defined conditions. Microscopic calibration was carried out as follows: 10 µl. of a *T. pallidum* suspension were placed on a slide and carefully covered with a 22 × 22-mm. cover slip so that the fluid was evenly distributed and devoid of air bubbles. In such a preparation the fluid was

0.01 ml.

$\frac{\quad}{4.84 \text{ cm.}^2} = 0.0207 \text{ mm.}$

thick. The diameter of the microscopic field, which must be determined for each microscope, was established as 0.41 mm. by using a slide micrometer. The area of each 0.41-mm. field was therefore 0.132 mm.² From these data the volume of fluid in one microscopic field was calculated to be 2.73×10^{-6} ml. An average of one organism per high dry field (hdf) (440 ×) thus represents a suspension containing 3.66×10^5 organisms/ml.

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Source of Aqueous Humour

Mature, healthy rabbits in which the serum was non-reactive to serological tests for syphilis were used to provide AH as an *in vitro* suspending medium for *T. pallidum*. The tests employed were the VDRL slide (U.S. Public Health Service, 1964), fluorescent treponemal antibody-absorption (FTA-ABS) (Staff, Venereal Disease Research Laboratory, 1968), and fluorescent treponemal antibody-1:5 (FTA-1:5) (Deacon, Falcone, and Harris, 1957). Within 24 hours after the first of a series of anterior chamber aspirations and refrigeration, AH was pooled and then divided into eleven 1-ml. samples for serial dilution with a stock suspension of virulent, motile treponemes.

Although serum from each rabbit had given negative results in the above serological tests for syphilis, AH from each donor was subjected to darkfield microscopy and direct FA examinations for the presence of spiral organisms. All examinations were found to be negative.

Dilution Scheme

Two-fold dilutions of *T. pallidum* in 1-ml. volumes of rabbit aqueous were carried out to 1:2,048 by using an initial 1-ml. treponemal suspension containing approximately 10.25×10^5 organisms. A twelfth tube of AH without *T. pallidum* and a thirteenth tube of the original test suspension were used as controls.

Quantitative Appraisal

Each *T. pallidum* dilution was mixed for 1 min. and the lower dilutions were checked by darkfield microscopy for uniformity of dispersion and number of organisms before sampling for direct immunofluorescent examinations; 10 μ l. were pipetted from each tube, placed on a slide, and dispersed over a circle 1 cm. in diameter. The procedure was repeated ten consecutive times for each corresponding tube dilution and for the two controls. The suspension was then allowed to dry in air, then immersed in acetone, and later stained by the direct FA technique (Venereal Disease Research Laboratory, 1967) by using rabbit anti-*T. pallidum* conjugated globulin produced in this laboratory (Lot No. 712227R2) that had common antibody removed by adsorption with Reiter treponemes (Deacon and Hunter, 1962; Meyer and Hunter, 1967). Both positive and negative controls were maintained during each "run" of slides.

Direct FA counts were made by random selection with 100 microscopic fields per slide by using a Leitz SM microscope with mercury lamp, HBO 200, ultra-violet light source equipped with BG-12 exciter and OG-1 barrier filters. The method of counting and a statistical analysis of the error inherent in this technique are to be reported by Artley and Clark (1969).

Results

All results represent a series of ten separate direct FA examinations (100 fields/examination) from each of the eleven dilutions and the two controls.

The Figure shows a four-cycle by four-cycle logarithmic graph with the ordinate represented by the average number of treponemes/hdf determined by random selection of 100 microscopic fields per slide. The abscissa represents the approximate number (thousands) of treponemes in 1 ml. of each of the eleven dilutions, which ranges from 500 to 512,000 organisms/1 ml. AH. The Figure shows that it is possible to relate the number of treponemes observed on the final FA slide to the number of organisms originally placed in each fluid sample. The straight line and associated equation shown in the Figure describe the line of best fit derived by applying the least square criterion to the logarithms of the average number of treponemes/hdf and the logarithms of the approximate number of organisms per ml. AH. Using direct immunofluorescent staining and rapid selection of 100 microscopic fields, we found that if one could demonstrate one treponeme per 100 fields (440 \times) then there were approximately 2,000 organisms/ml. AH. When dilutions of approximately 1,000 organisms/ml. AH were evaluated, one organism was demonstrated per 250 fields, and one per 500 fields with a dilution of approximately 500/ml. AH.

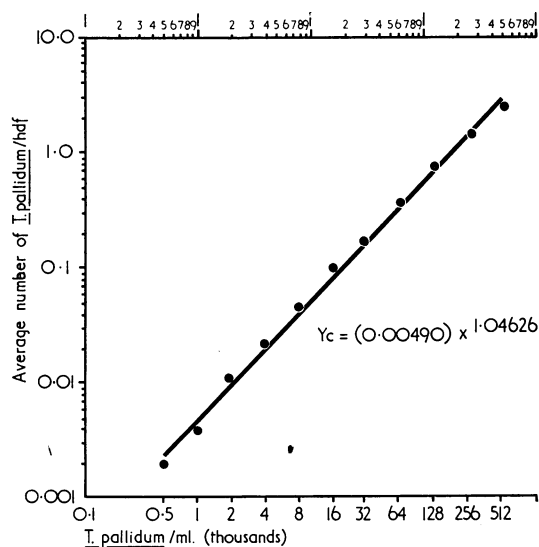


FIGURE.—Quantitative estimate of the number of spiral organisms/ml. aqueous humour as related to the number of organisms/hdf seen by direct immunofluorescent staining.
● Average of ten consecutive FA examinations.

To determine the number of organisms per ml. in a given sample of AH, one can multiply the average number of treponemes per field by the conversion factor 2.0×10^5 . For example, in the

above data, when there was one organism/100 fields, the average number of organisms per field was 0.01. Multiplying 0.01 by the factor 2.0×10^5 gives 2,000 treponemes/ml., which was the number present in the AH in this particular dilution. Under experimental conditions comparable to those in this study, the conversion factor is applicable when the graph in the Figure is not available or when one wishes to examine more than 100 fields per specimen.

Discussion

The present work establishes in a controlled-model system a quantitative relationship between the number of *T. pallida* present in AH and the number of such organisms that can be detected by immunofluorescent examination. This quantitative study provides a framework to aid in assessing future reports of the presence of spiral organisms in AH and other body fluids and tissues (Girard and others, 1967; Goldman and Girard, 1967, 1968; and Smith and Israel, 1967a, 1967b, 1968). Moreover, quantitation may aid in assessing response to therapy, a subject under active investigation in several centers.

Because of the difficulty of achieving exact enumeration of treponemes, we describe our study as quantitative with reservations. We used the direct enumeration method of Morgan and Vryonis (1938) which other workers have found satisfactory and which has become established by use over a long period in this laboratory. However, it might be best to regard with some caution our figures for numbers of organisms in the starting material, especially those for small numbers of organisms. In our study, the conversion factor for estimating from the final slide the number of treponemes/ml. AH was calculated as the average number of organisms per field multiplied by 2.0×10^5 . This factor holds good only for the microscope used in this study; it must be determined for each individual microscope, since field diameters vary with different instruments. It should be stressed that this conversion factor is derived from data based on the counting of 100 random fields per slide examined.

It was found in this study that 500 organisms/ml. AH yielded one FA-detectable organism in 500 high dry fields. We feel that routinely searching 500 fields per slide is approaching the limits of practicality even for research studies. Therefore, if for any reason one suspects that there are fewer than 500 treponemes/ml., one may take advantage of centrifugation to concentrate limited numbers of organisms.

Quantitative studies on the efficiency of centrifugation in microcapillary tubes have recently been

reported by Chandler and Cannefax (1969). These same investigators also quantitated the advantages that the immunofluorescent method can offer over darkfield techniques in the demonstration of *T. pallidum*. They further brought out that, although approximately 75 per cent. of the treponemes are lost during processing of the FA slides, the concentration of organisms within a small area of the slide and the stain's enhancement of the visibility of these organisms against the background more than adequately compensate for processing losses.

In the present study AH obtained from rabbits was used to dilute the stock solution of *T. pallidum*. Also, the treponemes were of the virulent Nichols strain, which is harvested from rabbits in which it is routinely passaged. This use of material from rabbit species may in some unknown way affect the applicability of our results to the human situation. Further, the *T. pallidum*-like organisms recently described in human AH may not be in the same metabolic state and conditions as the organisms we used in this study, which are harvested after 7 days of vigorous growth in rabbit testicular syphilomata.

Aqueous humour has been found to be a dynamic fluid, continuously entering and leaving the anterior chamber. Our data as a guide to the number of *T. pallida* that may be present therein thus apply only to the moment the paracentesis is performed. Our data are also based on the assumption that any organisms present in the AH are distributed throughout the fluid at random, so that any sample of aqueous is representative of the entire volume. Our data would not apply if for some reason the treponemes actively or passively congregated at certain sites within the AH, for such reasons as currents, affinity for lining structures, and small temperature variations.

It is reasonably well established that AH is formed in the anterior chamber by preferential accumulation of certain materials from the blood, and it has also been shown that particulates placed within the AH may pass out into the blood (Davson, 1962). Thus, as pointed out by Norins (in preparation), if treponemes are found within the AH, it is necessary to look "upstream" and "downstream" from the AH; organisms may be arriving from the blood and may be being sent out into the blood. Indeed, the older literature is replete with accounts of the infectivity of syphilitic blood and CSF (Chesney and Kemp, 1924; Frazier and Pian, 1948). Because organisms may enter or leave the AH at irregular intervals, and because organisms within the AH may or may not multiply at a fixed rate, our quantitation data apply only to the particular sample of AH under study, and do not by any means

predict the number of spiral organisms within the same eye even one day later. It must also be pointed out that the data in this report are applicable to the clinical reports of intraocular spiral organisms only in so far as some or all of the organisms are in fact *T. pallida*.

Summary

Recent demonstration of intraocular spiral organisms prompted the construction of an *in vitro* model to relate the number of *Treponema pallidum* observed by direct immunofluorescent staining of the aqueous humour to the approximate number of organisms per unit volume of the same specimen. Using a random selection of 100 microscopic fields ($440\times$) per smear, it was found in this particular model system that if one could demonstrate one treponeme/100 fields, then there were approximately 2,000 organisms/ml. aqueous humour. In the authors' hands, the conversion factor for determining the approximate number of treponemes per ml. aqueous humour was calculated as the average number of organisms per field multiplied by 2.0×10^6 , these factors being valid only for the microscope used in this study. Quantitation of *T. pallidum* may possibly be used to follow the evolution of infection and to assess the response to therapy.

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Études concernant le nombre de *T. pallidum* intra-oculaires; établissement d'un modèle *in vitro*

RÉSUMÉ

La récente démonstration d'organismes spirales intra-oculaires a incité à réaliser un modèle *in vitro* pour établir un rapport entre le nombre de *T. pallidum* observé par la coloration immunofluorescente directe de l'humeur aqueuse et le nombre approximatif d'organismes par unité de volume du même échantillon. Se servant d'une sélection de 100 champs microscopiques pris au hasard ($440\times$) par frottis, on a trouvé que si dans ce modèle particulier on pouvait mettre en évidence un tréponème par 100 champs, alors il y aurait à peu près 2.000 organismes par ml. d'humeur aqueuse. De ce travail, les auteurs ont calculé que le facteur de conversion pour déterminer le nombre approximatif de tréponèmes par ml. d'humeur aqueuse était la moyenne du nombre des organismes par champ multipliée par $2,0\times 10^6$, ce facteur étant valable seulement pour le microscope employé pour cette étude. Le nombre de *T. pallidum* pourrait, peut-être, être utile pour suivre l'évolution de l'infection et pour évaluer la réponse au traitement.